



Original scientific article

## Cryopreservation of genetically modified mouse strains: preserving valuable material, saving resources and reducing animal usage

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### Summary

Russell and Burch proposed the concept of the 3Rs, Replacement, Reduction and Refinement, in 1959 as a basis for ethical standards governing animal experimentation. Despite sincere attempts to implement these practices, increased use of genetically modified animals has created potential challenges to this framework. Specifically, genetically modified animals can be difficult to derive, and are therefore maintained continuously as a colony despite their transient experimental use.

As an alternative, the use of embryo or sperm cryopreservation provides a means to efficiently archive strains and eliminate unutilized strains, avoiding the birth and use of animals to maintain them. It also provides substantial reductions to cost and cage occupancy. Surprisingly, recent work by Zeller et al. (2017) indicates that only 56% of professionals working with laboratory animals are aware of cryopreservation as a technique for colony management.

This study shows that cryopreservation, within our institution, where the animal facility features a housing capacity of 3,000 cages and 10,000 to 15,000 mice, has allowed us to eliminate efficiently 115 unutilized genetically modified mouse strains during the past five years. This has i) liberated 19% of the animal house capacity, ii) prevented the birth of 21,189 unutilized mice, iii) lead to a saving of 382,800 € for our institution.

### Introduction

Russell and Burch's *Principles of Humane Experimental Technique* constitutes the basis of current ethical standards in animal experimentation (Russell and Burch 1959). They proposed the concept of the 3Rs, Replacement, Reduction and Refinement, which continues as a framework for minimizing discomfort to experimental animals. The 3Rs has been widely accepted by the international scientific community and has been embedded in international legislation regulating the use of laboratory animals (EU 2010).

Since its initial publication, numerous efforts have been made to apply the policy of 3Rs in animal experimentation (Festing 1995; Burch 1995). However, there are currently new challenges that must be addressed. The appearance of genetically modified animals (Gordon et al. 1980), has significantly increased the number of animal studies (Ormandy et al., 2009; Mazur et al. 2008; Critser and Mobraaten 2000). As a consequence, the number of animals used for experimental purposes has also continued

**Table 1.** Scientific procedures on living animals. Great Britain (1995-2015). Home Office. United Kingdom Government.

YEAR	1995	2000	2005	2010	2015
N° GMO* PROCEDURES	312,700	699,600	1,027,200	1,621,000	2,060,000
N° TOTAL PROCEDURES	2,709,600	2,714,700	2,896,200	3,724,700	4,140,000
PERCENTAGE GMO	11.5%	25.8%	35.5%	43.5%	49.8%

\*GMO: Genetically modified organism.

to grow (Ormandy et al. 2009, Hudson-Shore 2013; Hudson-Shore 2014). For example, in Great Britain, the annual statistics for the use of experimental animals (Home Office 2016) show a clear trend in this regard (Table 1). Thus, in 2015, procedures for the creation or breeding of genetically modified animals comprised half of the total number of procedures, with 91% of the animals used for the GMO production being mice.

The creation of genetically modified mouse strains is a considerable investment of time, money (it could need a minimum of 9 months and between 25,000 and 30,000 euros (Hagn et al. 2007)) and animals. The number of animals used is dependent on many factors (Nagy 2003) although there are guides that offer a good framework for estimation (Institute for Laboratory Animal Research (U.S.), Committee on Guidelines for the Use of Animals in Neuroscience and Behavioral Research, and National Academies Press (U.S.) 2003). By some calculations, generating founders and subsequent testing may involve the use of at least 70 mice (Buehr et al. 2003). However, the subsequent number of crosses required, combined with the potential number of offspring obtained, and the animals used in the basic phenotyping of the line (Fuchs et al. 2011), suggest the number can reach at least 250 animals for each transgenic line. More recently, the appearance of new programmable genome editing tools like the CRISPR/Cas9 system (Jinek et al. 2012) has allowed us to introduce different types of mutations more efficiently, requiring less time, less money and fewer animals, as it can be directly used on the background of interest.

Once experimental use of a transgenic line is finished, instead of germline cryopreservation, investigators often maintain a minimally sized colony as a means to preserve their transgenic lines. This 'minimum level' system not only produces ongoing waste of resources and animals, but does not comply with the recommendations for maintaining strains of genetically modified mice (The Jackson Laboratory 2009). This approach also increases the chances of fixing other mutations in the colony due to genetic drift (Taft et al. 2006; Zeldovich 2017), the silencing

of transgene expression (Kues et al., 2006) and may ultimately lead to the loss of the strain.

The possibility of cryopreserving mammalian embryos, and subsequently thawing them to recover live animals, was first described in mice (Whittingham et al. 1972). This approach offered the opportunity to archive and protect valuable strains such as genetically modified animals (Taft et al. 2006; Mobraaten 1986; Pomeroy 1991; Crabbe et al. 1993; Linder 2003; Wiles and Taft 2010), and has also been proposed as a means to manage colony size within animal facilities (Battey et al. 1999; Marschall and Hrabe de Angelis 1999; Abbott 2004; Agca 2012). Moreover, cryopreservation serves as a useful tool for 'reduction' within the 3R framework, by allowing the efficient elimination of strains of genetically modified mice that are not being used in research (Robinson et al. 2004; Osborne et al. 2009; Zeller et al. 2017).

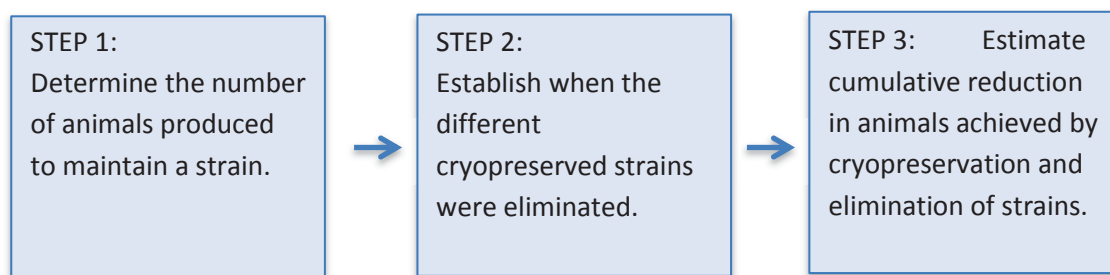
In conclusion, cryopreservation can help to save the investment that creation of a new line requires, avoid the risks of genetic drift and line loss, save resources (space and money), and most importantly reduce the number of animals, which is an ethical requirement in research.

In the current study we demonstrate how cryopreservation has allowed our institution to reduce the number of animals and save resources through the elimination of genetically modified mouse strains that were not actively enrolled on research studies.

## Materials and Methods

### Design

A descriptive observational study was carried out using data from strains cryopreserved by the Transgenic and Cryopreservation Laboratory of the Instituto de Neurociencias de Alicante CSIC-UMH between 2013 and 2017. The strains analyzed were housed in the RMG-SPF, a pathogen free animal facility within our institution, which has a maximum capacity of 3,000 cages and 10,000 to 15,000 mice, and a colony management program (ANIBIO®) in which all animals housed within the facility are registered.



The flow-chart describes the design of our study for determining the benefits of using cryopreservation based colony-size control:

#### STEP 1:

The number of animals produced to maintain a genetically modified mice strain was determined. We obtained for each cryopreserved strain, through the ANIBIO® program, the number of animals born the year before it was cryopreserved. The cryopreserved strains were classified as in-use or disuse, and the average number of animals born in a year to maintain them was then calculated and compared.

#### STEP 2:

We recorded when the different cryopreserved strains were eliminated. The elimination of a line is reflected in the reduction of animals and saving of resources in the years subsequent to elimination. Monitoring of eliminated strains was carried out until January 2018 through the ANIBIO® program.

#### STEP 3:

Finally, we estimated the cumulative animal and resource savings generated by cryopreservation and elimination of genetically modified mouse strains.

The animal reduction calculation was done using the value obtained per strain in the first step, although some eliminated strains were cryopreserved the same year that were created or established in the RMG facility and so, due to the absence of previous demographic records, the average number of mice born per strain in disuse was used for the calculation.

For the resource savings calculation, it was necessary to determine the average number of cages used to maintain a minimal colony of genetically modified mice, and the annual cost of maintaining a mouse cage. However, in our institution, the number of cages used per transgenic line is not registered in the ANIBIO® program. We therefore relied on our historical breeding experience, and on data from literature searches to determine the space and money required to keep a minimal colony of genetically

modified mice alive; this provided a more comprehensive view of what happens in other facilities. For the resource savings calculation, the cost of maintaining the Transgenic and Cryopreservation Laboratory was taken into account. This is about 90.000€ per year, including salaries, culture media, consumable materials, equipment amortization, animals, repairs and calibrations.

### Animals

The mice used for the study were born and housed in the RMG-SPF animal facility in the Instituto de Neurociencias de Alicante CSIC-UMH, under stable, controlled environmental conditions, according to standards specified by national regulations. There was a 12-hour light/dark cycle (lights on at 8:00), constant temperature of  $22\pm1^{\circ}\text{C}$ ,  $55\pm5\%$  relative humidity, and animals had free access to food and water. The procedures for embryo and sperm cryopreservation of the strains performed by our laboratory were reviewed and approved by the UMH Project Evaluation Board. All genetically modified mouse strains that were cryopreserved were part of research projects approved by the Spanish competent authority.

### Data analysis

The data was statistically processed using the IBM® SPSS® Statistics Version 23.0 program.

Due to high variability, the data obtained did not follow a normal distribution and showed a high standard deviation. To calculate the average number of animals born per year per line both in-use or in disuse, we used the interquartile range (25th to 75th percentile) for analysis. In order to be able to compare those averages, a two-sample Student's t-test was used. The differences were regarded as significant when  $P < 0.05$ .

## Results

### Number of animals born per year per cryopreserved line of genetically modified mice.

The average number of mice born per year per cryopreserved line was  $105.8 \pm 30.7$  for in-use strains and  $54.5 \pm 16.1$  for disuse strains ( $P < 0.05$ ).

### Cryopreserved strains and elimination of disused strains.

In the 2013-2017 period, the Transgenic and Cryopreservation Laboratory of the Instituto de Neurociencias de Alicante CSIC-UMH cryopreserved 215 strains of genetically modified mice. Of these, 58 corresponded to external services and 157 were housed in the RMG Facility.

By January 2018, of the 157 strains housed in the RMG facility, 115 (73.2%) had been completely eliminated after they were no longer in use, while 42 strains remained alive. Of the 115 eliminated strains, 91 (79.1%) were eliminated in the same year they were cryopreserved and 24 (20.9%) were eliminated in subsequent years.

Regarding the exact moment of elimination, 29 strains were eliminated in 2013, 13 in 2014, 28 in 2015, 21 in 2016 and 24 in 2017 (Table 2).

### Cumulative reduction of animals by cryopreservation and elimination of disused strains.

In 2013, 29 cryopreserved strains were eliminated because they were not being used. These strains produced the year before their cryopreservation 1,947 animals, so we could estimate that keeping them alive in that state would have generated this number of animals each year. Therefore, cryopreservation and subsequent elimination had prevented 1,947 animals being born each year.

Following the same reasoning, in 2014, 13 unused cryopreserved strains were eliminated, therefore preventing 693 animals being born in 2015 and in subsequent years.

In 2015, 28 unused cryopreserved strains were eliminated, and this prevented in 2016 and afterwards, 1,398 animals being born every year.

In 2016, 21 cryopreserved unused strains were eliminated and therefore, 1,388 animals were prevented from being born in 2017, and the same number of animals would have been avoided in 2018.

**Table 2.** Cryopreserved mice strains by the Transgenic and Cryopreservation Laboratory of the Instituto de Neurociencias CSIC-UMH (2013 - 2017).

YEAR	CRYO STRAINS	RMG FACILITY STRAINS	ELIMINATED STRAINS & YEAR OF ELIMINATION		NOT ELIMINATED STRAINS
2013	35	32	29	2013	2
			1	2015	
2014	60	32	13	2014	9
			1	2015	
			5	2016	
			4	2017	
2015	43	40	26	2015	7
			5	2016	
			2	2017	
2016	37	31	11	2016	14
			6	2017	
2017	40	22	12	2017	10
TOTAL 2013-2017	215	157	29	2013	42
			13	2014	
			28	2015	
			21	2016	
			24	2017	

**Table 3.** Cumulative reduction of animals by cryopreservation and elimination of disused strains (2013-2017).

		YEAR OF ELIMINATION				
REDUCTION OF ANIMALS PER YEAR	YEAR	2013	2014	2015	2016	2017
	2014	1,947				
	2015	1,947	693			
	2016	1,947	693	1,398		
	2017	1,947	693	1,398	1,388	
	2018	1,947	693	1,398	1,388	1,712
	TOTAL/YEAR	9,735	2,772	4,194	2,776	1,712
TOTAL REDUCTION		21,189				

Finally, in 2017, 24 strains that had been cryopreserved were eliminated, which as a result, prevented the birth of 1,712 animals in 2018.

Overall, cryopreservation of strains in the period 2013-2017 and the subsequent elimination of some of them will have produced a cumulative reduction of 21,189 animals by 2018 (Table 3).

### Cumulative resource savings (space and money) generated by cryopreservation and elimination of disused mouse strains

Based on our historical breeding experience, and our literature searches, we determined that maintaining a minimal colony of genetically modified mice would require the use of 4 to 5 cages per month (Landel 2005). This value was further supported by the average number of animals born annually within disused strains (54.5 animals, section 3.1). Thus, including 1 or 2 breeding cages, cages with stock animals, weaning etc., the use of an average of 5 cages per month to maintain a disused strain is a reasonable number and therefore has been used for calculating resource savings. As for the cost of maintaining these cages,

we can use the average annual value of 480 € per cage (The Jackson Laboratory 2009).

Therefore, keeping alive the 115 strains eliminated in the study period would have required the use of 575 cages and occupied 19% of the total space of the RMG-SPF Animal Facility. In economic terms, we can estimate that the use of cages to keep alive the 115 disused strains eliminated would have involved a cost of 832,800 € by the end of the study period. Taking into account the cost of cryopreservation (90,000 € per year for maintaining the Transgenic and Cryopreservation Laboratory), we estimate a total cost savings in this period of 382,800 € (Table 4).

### Discussion

The results of this study show how the establishment of a cryopreservation laboratory eliminates a significant number of genetically modified mice strains that are not being used and that otherwise would have had to be kept alive so as not to be lost. This has a clear impact on the management of animal facilities, by improving the use of resources, especially saving

**Table 4.** Cumulative cost savings by cryopreservation and elimination of disused strains (2013-2017).

		YEAR OF ELIMINATION				
COST SAVINGS PER YEAR	YEAR	2013	2014	2015	2016	2017
	2014	69,600				
	2015	69,600	31,200			
	2016	69,600	31,200	67,200		
	2017	69,600	31,200	67,200	50,400	
	2018	69,600	31,200	67,200	50,400	57,600
	TOTAL/YEAR	348,000	124,800	201,600	100,800	57,600
CYOPRESERVATION COST		- 90,000	- 90,000	- 90,000	- 90,000	- 90,000
TOTAL COSTS SAVINGS		382,800 €				



space and money. The approach undertaken by our laboratory in the last 5 years resulted in savings of approximately 382,800 € and 19% of animal space which would otherwise have been wasted on maintaining strains without any experimental interest.

Keeping strains of mice in disuse alive not only wastes resources and animals, but, as our study shows, colonies usually remain at “minimum levels”, and this can be the source of some serious and irreversible problems. Thus, our results indicate that the average number of animals born per year in the disused strains is approximately half that in the strains used in research

Having fewer animals may promote genetic bottlenecks in which the chances of fixing mutations that arise randomly by genetic drift increase, but also increases the chances of strain loss due to reproductive failures or errors in animal management. In the 2013-2017 period this was the situation for 17 strains in our facility. One of them lost the phenotype because of a promoter methylation and 16 strains were close to disappearing due to breeding cessation and had to be rescued from extinction by *in vivo* sampling of epididymal sperm and IVF (Del Val and Robledano 2013). Any of these problems can irreparably lead to a loss on the initial investment associated with establishing a strain (up to 250 animals per strain), and therefore the archiving through cryopreservation prevents that investment from being lost and having to be repeated.

In addition, cryopreservation has a more important benefit for animal welfare by facilitating humane elimination of disused strains. In our institution 115 of the 157 cryopreserved strains in the 2013-2017 period were eliminated. This resulted in a cumulative reduction of 21,189 animals by the year 2018, which otherwise would have been born if these strains had been kept alive.

In short, if we put these results in the context of our medium sized animal facility, which features a maximum capacity of 3,000 cages and housing for 10,000 to 15,000 mice, we can see that the figures of animal reduction and resource saving are considerable. This gives an idea of the potential that cryopreservation would have if applied in a systematic way in all research centres to reduce the number of experimental animals being used, and especially to curb the relentless rise in the number of animals used in the creation and breeding of genetically modified strains.

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